Journal of Pharmacological and Toxicological Methods 123 (2023) 107296

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Optimization of bioanalysis of dried blood samples

Elham Ataei Alizadeh [a], Georg Rast [a], Chris Cantow [b], Jessica Schiwon [a], Florian Krause [a], Guido R.Y. De Meyer [c], Pieter-Jan Guns [c], Brian D. Guth [a], Michael Markert [a,*]

[a] Department of Drug Discovery Sciences, General Pharmacology Group, Boehringer Ingelheim Pharma GmbH & Co KG, Germany
[b] Department of Drug Discovery Sciences, Bioanalysis Team, Boehringer Ingelheim Pharma GmbH & Co KG, Germany
[c] Laboratory of Physiopharmacology, University of Antwerp, Antwerp, Belgium

ARTICLE INFO

Keywords:
Safety pharmacology
In-vivo blood sampling
PK/PD
Dog
Bioanalysis
Drug concentration

ABSTRACT

Introduction: Pharmacokinetic/pharmacodynamic modelling has emerged as a valuable technique for understanding drug exposure and response relationships in drug development. Pharmacokinetic data are often obtained by taking multiple blood samples, which may disturb physiological parameters and complicate study designs. Wearable automatic blood sampling systems can improve this limitation by collecting dried blood samples at programmable time points without disrupting cardiovascular parameters. It is the objective of this study to evaluate the bioanalysis of DBS in comparison to conventional blood sampling techniques and to optimize the recovery of various compounds spiked into canine blood dried on filter paper tape.

Methods: Incubated blood samples from Beagle dogs were spiked with 16 different compounds and half of the whole blood sample was centrifuged to obtain plasma. After the dried blood sample drops were dried, liquid chromatography-mass spectrometry methods were used to analyze the samples. The study explored different anticoagulants, sample preparation methods and technical approaches to best determine the compound concentrations in dried blood samples.

Results: With the two anticoagulants tested and using the optimized sample preparation methods and technical approaches we employed, the bioanalysis of dried blood samples can provide equivalent results to conventional blood sampling techniques.

Discussion: Automated blood sampling systems have the potential to provide increased numbers of blood samples, providing substantially more Pharmacokinetic data within safety pharmacology studies without disrupting physiological parameters. They can provide a viable alternative to traditional methods of obtaining blood for various other types of studies or analyses.

1. Introduction

Successful drug development requires proof of therapeutic efficacy and patient safety (Guns et al., 2020). Pharmacokinetic/pharmacodynamic (PK/PD) modelling is an approach that is being used increasingly in drug development and safety pharmacology to relate drug exposure to a given effect (Morgan et al., 2018; Peck et al., 1992; Van Der Graaf & Benson, 2011). European Medicines Agency (EMA)/ Committee for Medicinal Products for Human Use (CHMP) guidance on the use of pharmacokinetics and pharmacodynamics in the development of antimicrobial medicinal products provides recommendations for using PK/PD modelling to inform regulatory decisions about clinical trial design, conduct, analysis and reporting (EMA/CHMP, 2016). To assess the relationship between drug exposure and response, Food and Drug Administration (FDA) guidance on exposure-response relationships and pharmacogenomics recommends pharmacokinetic (PK)/pharmacodynamic (PD) modelling (FDA, 2022).

PK/PD modelling and obtaining a comprehensive understanding of drug effects throughout the time a drug is absorbed, distributed and eliminated from the body, requires that multiple blood samples need to be taken concurrently with physiological measures (Rajman, 2008; Workman, 2002). Blood sampling is basically an easy procedure,

Abbreviations: Biopharmaceutics Classification System, BCS; Committee for Medicinal Products for Human Use, CHMP; Concentration in blood, Cb; Compound Concentration, CC; Concentration in plasma, Cp; Dried Blood Samples, DBS; Ethylenediaminetetraacetic acid, EDTA; European Medicines Agency, EMA; Food and Drug Administration, FDA; Minimum Detectable Difference, MDD; Pharmacokinetic, PK; Pharmacodynamic, PD; Whole Blood, WHB.

* Corresponding author at Drug Discovery Science, Boehringer Ingelheim Pharma GmbH & Co. KG, Birkendorfer Str. 65· 88400 Biberach an der Riß, Germany.
E-mail address: michael.markert@boehringer-ingelheim.com (M. Markert).

https://doi.org/10.1016/j.vascn.2023.107296
Received 27 June 2023; Received in revised form 13 July 2023; Accepted 16 July 2023
Available online 22 July 2023

however, in the conscious animal, the interaction of technical staff with the animal to obtain a sample is associated with short-term, but often marked changes in hemodynamic parameters (Alizadeh et al., 2023). Traditional toxicology studies often include extensive blood sampling and limited physiological information, whereas safety pharmacology studies have extensive high-quality physiological data (heart rate, blood pressure, and ECG), but have very limited plasma samples, or use a satellite cohort technique to obtain pharmacokinetics (Musteata, De Lannoy, Gien, & Pawliszyn, 2008; Van Groen et al., 2020). In safety pharmacology studies, the number of blood samples taken is therefore kept to a minimum in order to avoid disturbing the parameters that are being measured. Increasing the number of blood samples taken, without disturbing the physiological parameters of interest, would be highly beneficial, as it would allow a far better assessment of the relationship between drug effects and drug concentration. Thus, a device enabling more blood sampling time points while avoiding a disturbance of cardiovascular parameters would provide a major advantage by reducing the need for additional PK studies and allowing for a more detailed PK/PD modelling (Rajman, 2008; Workman, 2002).

Wearable automatic blood sampling systems (e.g. Fluispotter®, Fluisense ApS, Denmark) have recently become available. By using paper tape and programmable time settings, these devices enable serial sampling with high accuracy and precision blood volumes to be collected as dried blood samples (DBS) during pharmacological studies (explained below) (Adhikari et al., 2020). The device collects whole blood dried on the filter paper tape, with citrate as an anticoagulant. The bioanalysis of compound concentration using such whole blood dried onto filter paper has not been well defined nor optimized. Therefore, the current ex vivo study was conducted to compare the recovery of various types of compounds spiked into canine blood with blood samples dried onto the filter paper tape. This entailed using and comparing various procedures for extracting the dried blood from the paper.

Despite the convenience and minimal invasiveness of sampling dried blood spots, it is important to consider the potential challenges and differences in comparison to conventional plasma measurements (Skogstrand et al., 2008; Spooner, Lad, & Barfield, 2009; Wilhelm, Burger, & Swart, 2014). For instance, the blood-to-plasma ratio depends on the hematocrit, which can lead to an inaccurate calculation of plasma drug concentration (Velghe, Delahaye, & Stove, 2019). Another challenge associated with DBS, determining the correlation between plasma and DBS drug concentrations is also important. Drying may lead to the binding of some compounds to the matrix, resulting in a loss in measurement accuracy (Keustermans, Hoeks, Meerding, Prakken, & De Jager, 2013). It is crucial to ensure that plasma concentrations are accurately and reliably correlated. Therefore, optimizing measurement protocols and sample preparation may be necessary to ensure accurate and reliable measurements.

The study goal was to provide an optimized bioanalytical assessment of DBS, in comparison to measuring compound concentration in blood or plasma using conventional manual blood sampling techniques.

2. Methods

In this article, the compound concentration from dried blood samples gathered by an automatic blood sampler device has been investigated with regard to different aspects of sample preparation methods, technical approaches, different types of compounds, differences between dry blood and conventional methods and different anticoagulants.

2.1. Device design

For the purposes of this study, the Fluispotter automatic blood sampler (REF: F10100) has been selected to be a reference device for collecting DBS. Thus, only filter paper used in this device was used for this validation. With Fluispotter, serial predefined micro-sampling of up to 10 μL DBS (in this study 10 μL was used) can be performed unattended for up to 48 h, using a strip of PerkinElmer 226 filter paper, at user-defined intervals (Adhikari et al., 2020; Ollerenshaw, Schrøder, & Velschow, 2022). To prevent the coagulation of blood inside the cartridge, the blood is mixed with a 4% sodium citrate flushing solution (1:9 dilution). By using the control unit, sampling times and sample volumes are defined and logged.

2.2. Study design

Blood samples were taken from Beagle dogs using venipuncture and were used as 1 mL whole blood aliquots (WHB, Fig. 1.A). The samples were then spiked with one of 16 compounds at a final concentration of 1 μM and incubated for 15 min at 37 °C. Eight of the compounds were from Boehringer Ingelheim's research pool, whereas the other eight are public domain reference compounds. The selection of compounds was made such that all four main Biopharmaceutics Classification System (BCS) classes were represented (see Table 1). After incubation, half of the whole blood was centrifuged to obtain plasma for further bioanalysis (Plasma of WHB, Fig. 1.B). The whole blood was used after incubation to produce 9 drops on the paper strip (DBS, Fig. 1.C). In triplicate, as references, 10 μL of WHB and Plasma from WHB were then pipetted onto a bioanalytical 96-well plate, sealed and stored overnight in a cooler. During this time, the DBS drops were allowed to dry.

The Protein Precipitation method was used for sample preparation (Burgess, 2009). Seventy μL of the organic phase, acetonitrile, mixed with methanol in a 50/50 ratio was added to 10 μL of plasma, whole blood or samples derived from the Fluispotter samples. Chromatography–mass spectrometry (LC–MS) methods were used to determine the compound concentrations in the samples (Choi et al., 2019). We normalized results in percentages of the WBS, which was considered to be the gold standard for this study.

In order to predict the compound concentration in vivo, it is necessary to transform the DBS results into WBS. Thus, we have calculated the compound concentration of "Plasma from WHB" (plasma after incubation and centrifugation of blood, which simulates body compound concentration) and divided it by WHB compound concentration to measure the ratio between them. This "extrapolates" the results from DBS to the plasma from WBS. Fig. 2 shows the scheme of this concentration in plasma/concentration in blood (Cp/Cb) calculation.

To determine if the anticoagulant used affects WBS, we compared the mean percentages of compound concentrations of WBS, and plasma from WBS with Ethylenediaminetetraacetic acid (EDTA) and Citrate as the most popular anticoagulants (Chen, Fong, & Chiang, 1999; Hennø et al., 2017; Lee & Arepally, 2012). Tubes with EDTA were coated with EDTA K3E/1.2 mL on its wall, however tubes with Citrate maintained Citrate 9NC/3.8 mL as a liquid.

2.3. Technical standardization and optimization of bioanalytical samples

2.3.1. Blood samples

S-Monovette® K3 EDTA 1.2 mL vials coated with EDTA were used for the EDTA anticoagulant. To collect blood samples for Citrate anticoagulant, S-Monovette® PFA, Citrate 3.8% 3.8 mL vials were used.

2.3.2. Vials

Dried blood samples were analysed by extracting the analyte from the paper using vials containing small beads made from different materials. These spheres were added to break up the paper during shaking. Prior to conducting the study, the optimal vial and sphere characteristics for fragmentation of DBS and liquid extraction were selected. These vials are made for the homogenization of tissue samples. This preliminary evaluation showed that the best results were obtained using a 2 mL vial "lysing with hard tissue homogenised spheres CK28-15mL".

2.3.3. Liquid extraction

The goal of the procedure was an extraction of the analyte to the

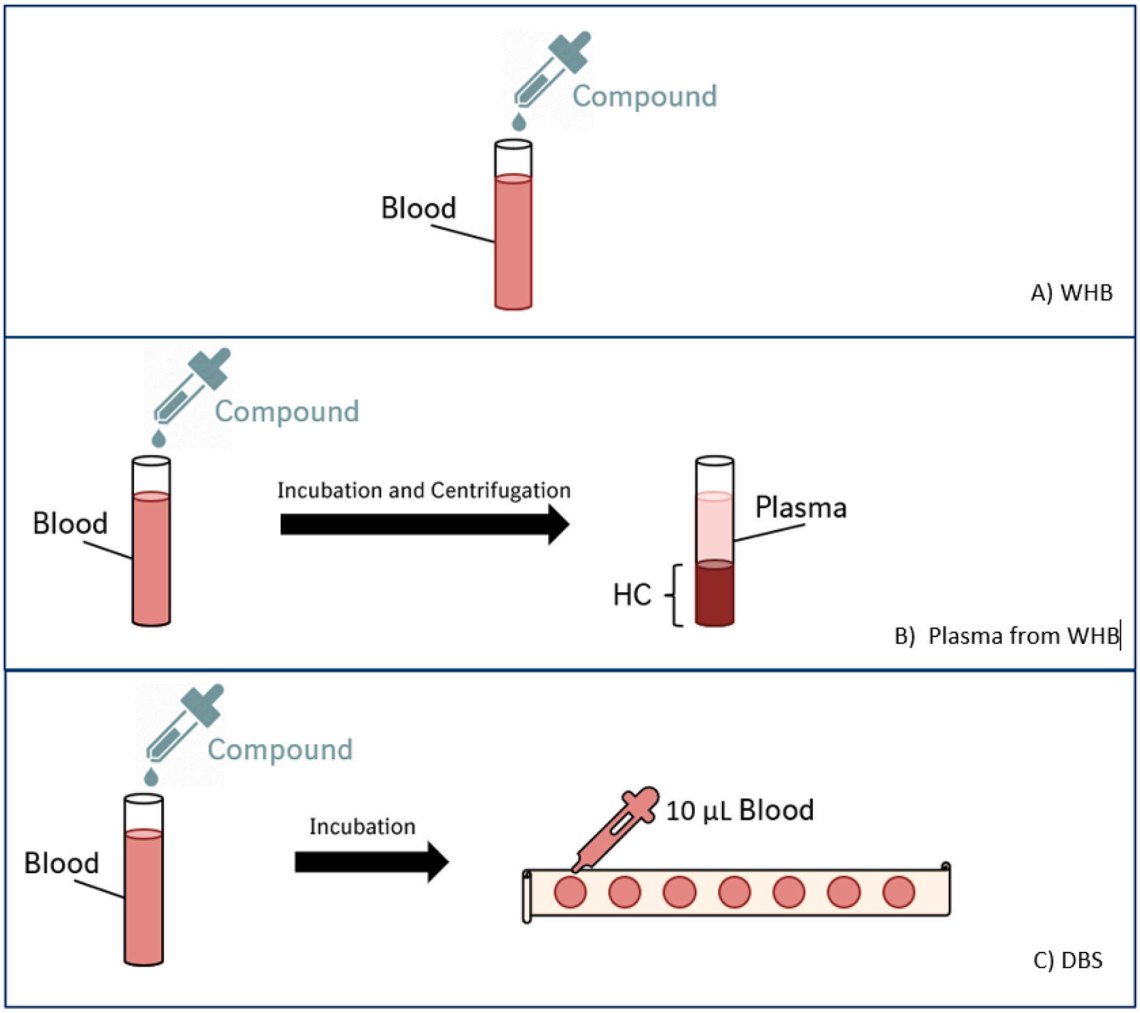


Fig. 1. Sample preparation methods for analysis. A) spiked compound into fresh whole blood (WHB), B) compound was spiked into fresh whole blood and incubated for 15 min before centrifugation and extraction of the plasma part was carried out, C) compound was spiked into fresh whole blood, and after 15 min of incubation, 10 µL of blood were pipetted on paper tape and allowed to dry overnight. "Plasma from WHB" is considered as 100% reference.

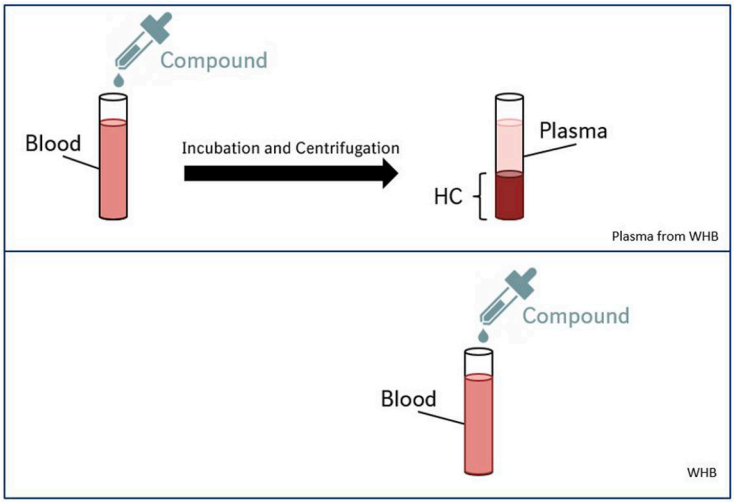


Fig. 2. Cp/Cb calculation setup. Cp) is compound concentration of spiked compound into whole blood and incubated for 15 min before centrifugation and extraction of the plasma part in order to measure the compound concentration, Cb) is compound concentration in spied compound into whole blood in order to measure the compound concentration.

greatest extent possible from the paper/solid part after shaking and centrifugation to provide a liquid sample for bioanalysis.

Three different solutions (Neutral, Acidic and basic) were used to extract the DBS samples and we tested each solution three times. Each vial contained 300 μL of each liquid solution which means the dilution factor was 30-fold for the actual compound concentration. Below is a list of solutions tested:

Neutral: ACN/MeOH/H_2O (37.5/37.5/25%) - > pH value ~7

Acidic: ACN/MeOH/H_2O (37.5/37.5/25%) + 2% HCOOH - > pH value ~3

Basic: ACN/MeOH/H_2O (37.5/37.5/25%) + 0.5% NH_4OH - > pH value ~10

2.4. Selection of reference compounds

Extraction of compounds relies on their solubility, which is influenced by both water and organic solvents. Often, the solubility of research compounds depends on their charge state, which can be affected by pH and pKa values. Therefore, optimal pH should promote a high proportion of charged molecules, resulting in greater solubility. In this study, we selected compounds of different chemical classes, based on the BCS (Chavda, Patel, & Anand, 2010; Reddy & Karunakar, 2011), as well as compounds having different pKa values (Gross, Seybold, & Hadad, 2002) and PH solubility (Shieh, Liu, Wu, & Lee, 2007). Table 1 summarizes the list of compounds, their BCS classification and their PKa values.

2.5. Data analysis

To compare the samples of "Plasma from WHB" and DBS for each compound, the compound concentrations of DBS were first multiplied by 30 as the dilution factor of liquid solution for extracting the DBS (see above). Then, the results were normalized with plasma from WHB being 100%. The mean of compound concentration of all animals' "Plasma from WHB" has been considered for each compound as a simulation of manual blood sampling during pharmacological studies.

One way ANOVA with Dunnett's correction was calculated to check for significant differences between each DBS and "Plasma from WHB". Using a minimum detectable difference (MDD) of compound concentration between DBS and "Plasma from WHB" the smallest difference between two samples was detected with a 95% level of confidence. Statistical calculations for the biological replicates ($N = 4$) are conducted first by calculating the mean of the technical replicates ($n = 3$). To compare the two samples, mean and SE differences were calculated between DBS and "Plasma from WHB".

R programming was used for statistical testing and analysis and Tibco Spotfire software (TIBCO Spotfire®) was used to display individual results.

3. Results

3.1. Anticoagulants: EDTA versus citrate

The vials with Citrate anticoagulant contained 3.8% liquid buffer whereas the vials with EDTA were coated with EDTA without liquid; thus, the whole blood was diluted slightly with Citrate liquid. Despite this dilution when using Citrate, no difference in the compound concentration has been observed from the bioanalytical perspective. In Fig. 3, compound concentrations in EDTA and Citrate samples are compared based on the average of all four animals and all compounds.

3.2. Testing a single concentration

Three different liquid solutions at three different pH values were tested to extract the DBS. The percentage results of each compound (mean of four animals) in each of the three liquid solutions are shown in Table 2. The concentrations of compounds (CCs) with the best match with conventional samples have been highlighted in green and selected for statistical comparison. Indeed, it is recommended to evaluate and select the optimal conditions for a given compound prior to conducting a study.

Table 3 shows differences between the selected DBS results (after

Table 1
List of tested compounds and their biochemical characteristics, pKa variations (Gross et al., 2002), pH and solubility.

Compound Name	BCS Classification	pKa	Solvent	Solubility (mg/mL)
Metoprolol	Cluster 1	9.6	NaOH solution pH 13.0 0.1 M	18.7
Paracetamol	Cluster 1	no pKa-value between pH 2–12		
Moxifloxacin	Cluster 1	8.6; 6.2		
Glibenclamide	Cluster 2	no pKa-value between pH 2–12	SörensenBuf pH 10.0 Phos. buf. pH 7.4 0.1 M McIlvaineBuf pH 3.0	0.26 0.008 0.003
Bicalutamide	Cluster 2	no pKa-value between pH 2–12		
Aceclofenac	Cluster 2	3.2		
Cimetidine	Cluster 3	7.0	NaOH solution pH 13.0 0.1 M	5.1
Bifonazole	Cluster 4	5.6		
Compound 1	Cluster 1	8.5	McIlvaineBuf pH 3.0 McIlvaineBuf pH 7.4	>5.000 1.300
Compound 2	Cluster 1	3.8	McIlvaineBuf pH 6.8 McIlvaineBuf pH 3.0	>1.000 > 1.000
Compound 3	Cluster 2	7.6	SoerensenBuf pH 10.0 McIlvaineBuf pH 7.4 McIlvaineBuf pH 3.0	0.070 < 0.001 < 0.001
Compound 4	Cluster 2	4.1	SoerensenBuf pH 10.0 McIlvaineBuf pH 7.4 McIlvaineBuf pH 3.0	4.800 < 0.001 < 0.001
Compound 5	Cluster 3	7.2	SörensenBuf pH 10.0 McIlvaineBuf pH 7.4 McIlvaineBuf pH 3.0	1.600 0.100 0.070
Compound 6	Cluster 3	8.5; 9.2	SoerensenBuf pH 10.0 McIlvaineBuf pH 6.8 McIlvaineBuf pH 3.0	0.270 > 10.000 > 10.000
Compound 7	Cluster 4	3.1; 5.7	McIlvaineBuf pH 7.4 McIlvaineBuf pH 3.0	<0.001 0.400
Compound 8	Cluster 4	8.6	SörensenBuf pH 10.0 McIlvaineBuf pH 6.8 McIlvaineBuf pH 2.2	0.200 0.030 0.070

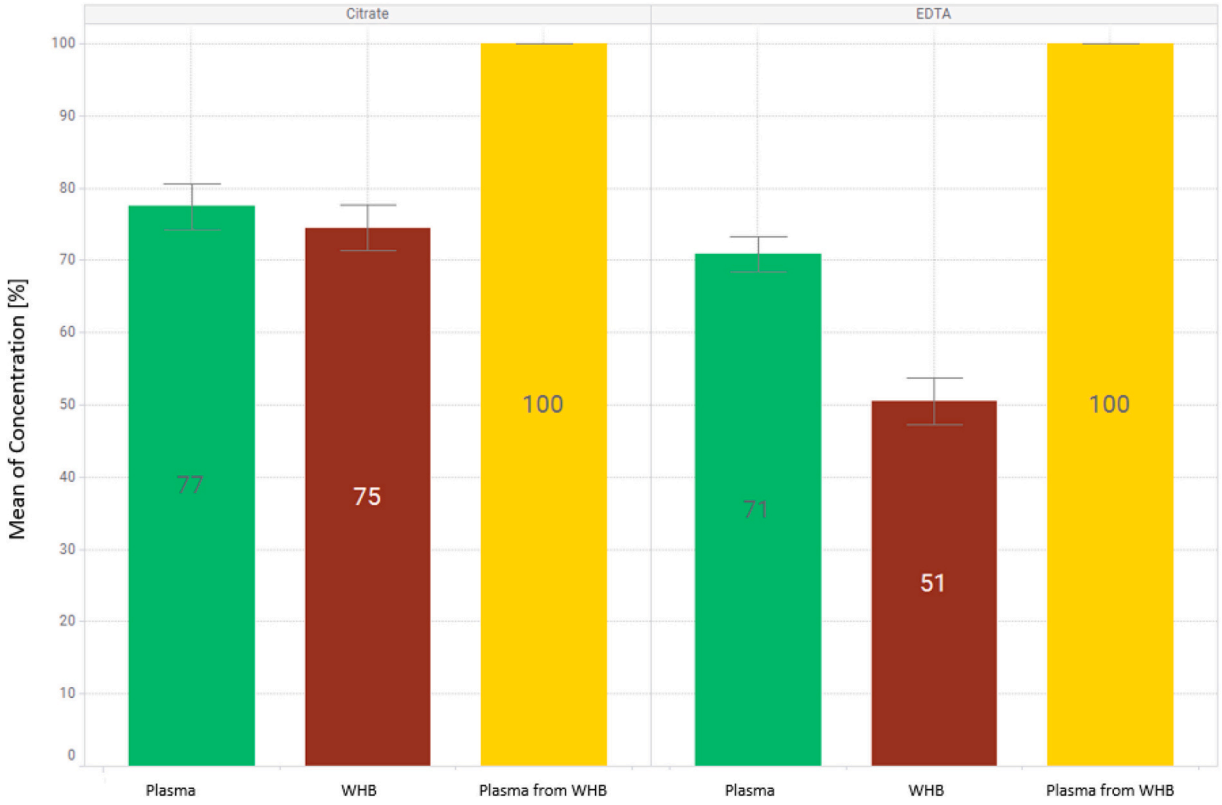


Fig. 3. Mean (four animals) of all compounds concentrations in EDTA and Citrate anticoagulant, between samples "WHB" and "Plasma from WHB". Values have been normalized by percentages, based on "Plasma from WHB" as the conventional sample of 100%. *P*-value less than [0.001] appears as '***', between [0.001, 0.01] appears as '**', between [0.01, 0.05] appears as '*', and when *p*-value >0.1, there is N/S.

multiplying with Cp/Cb ratio) and compound concentration of plasma from "Plasma from WHB", for each compound. The mean concentration of compounds in the blood of Beagle dogs, was normalized by percentages for better comparison. As a simulation of conventional bioanalysis, the mean of "Plasma from WHB" is considered to be a reference of 100%. For more information about the exact compound concentrations, see the supplementary material.

Fig. 4 illustrates a representative compound per BCS cluster. Graphs for the other compounds are found in the supplementary material. Detailed comparisons of DBSs and conventional systems for all individual 16 tested compounds are also shown in the supplementary material.

3.3. Testing of different concentrations

Four different compound concentrations (50, 100, 500, and 1000 nM) with the three different liquid solutions were compared with extracted DBS in order to verify that the same components were being extracted and measured in synthetic DBSs and conventional samples. A comparison of compound concentrations of DBS after multiplying with the Cp/Cb ratio and compound concentrations of "Plasma from WHB" for each reference compound in different concentrations was carried out.

Fig. 5 shows the mean of all compounds in different concentrations to validate the DBS in different compound concentrations. As in whole blood, DBS compound concentrations were measurable at different compound concentrations, and Cp/Cb remained constant. A detailed comparison of the compound concentrations in DBSs and conventional systems for each individual compound is shown in the supplementary material.

4. Discussion

4.1. Fluispotter dried blood samples

The automatic blood sampler collects an accurate and precise volume of whole blood which can be chosen between 3 and 10 μL and this is stored as a drop on filter paper. The filter paper is 100% cotton with pH 5.7–7.5. This filter paper is commonly used in laboratory settings due to its high quality. The combination of its high wet strength, chemical resistance and low extractable content makes it an attractive choice for a variety of applications, including collecting and preserving samples (Ahmad, She, & Kraatz, 2019; Hang et al., 2017). On the basis of this evaluation, it appears that the filter paper is amenable to the dried blood sample procedure and the blood can be effectively recovered from the paper after drying when using an optimized technical approach.

4.2. Citrate anticoagulant

Blood clotting is commonly prevented using anticoagulants in in-vivo studies and research settings. Citrate and EDTA anticoagulants are readily available and commonly used in research settings and in-vivo studies (Eldjarn, Andersen, Forfang, Hagve, & Rootwelt, 2011). Citrate is also used as an anticoagulant in the Fluispotter automatic blood sampler. Compound concentrations were not found to differ when comparing Citrate and EDTA as the anticoagulant in this in vitro study. However, pros and cons must still be considered, such as EDTA's irreversibility versus citrate's reversibility. In most cases, EDTA is used to

Table 2
List of compounds and their concentration (CC) with each liquid solution. The heist CC has been highlighted in green.

Compound Name	BCS Classification	pKa	CC* with Neutral liquid	CC* with Acidic liquid	CC* with Basic liquid
Metoprolol	Cluster 1	9.6	104%	119%	105%
Paracetamol	Cluster 1		77%	72%	52%
Moxifloxacin	Cluster 1	8.6 ; 6.2	102%	125%	108%
Glibenclamide	Cluster 2		137%	27%	91%
Bicalutamide	Cluster 2		136%	4%	66%
Aceclofenac	Cluster 2	3.2	123%	11%	80%
Cimetidine	Cluster 3	7.0	99%	106%	94%
Bifonazole	Cluster 4	5.6	100%	5%	83%
Compound 1	Cluster 1	8.5	78%	88%	52%
Compound 2	Cluster 1	3.8	85%	74%	85%
Compound 3	Cluster 2	7.6	110%	11%	72%
Compound 4	Cluster 2	4.1	69%	33%	68%
Compound 5	Cluster 3	7.2	168%	6%	81%
Compound 6	Cluster 3	8.5 ; 9.2	80%	162%	130%
Compound 7	Cluster 4	3.1 ; 5.7	81%	86%	80%
Compound 8	Cluster 4	8.6	97%	23%	66%

Table 3
List of compounds and their biochemical characteristics.

Compound Name	BCS Classification	pKa	MDD	Mean difference	SE difference	P-Value
Metoprolol	Cluster 1	9.6	15.28%	3.62%	1.31%	0.4179
Paracetamol	Cluster 1		21.48%	22.39%	3.46%	0.0893
Moxifloxacin	Cluster 1	8.6; 6.2	7.03%	1.80%	0.88%	0.0001
Glibenclamide	Cluster 2		20.90%	7.89%	2.04%	0.0001
Bicalutamide	Cluster 2		10.90%	35.87%	1.77%	0.0032
Aceclofenac	Cluster 2	3.2	18.37%	19.98%	2.00%	0.0001
Cimetidine	Cluster 3	7.0	22.37%	1.01%	0.95%	0.5493
Bifonazole	Cluster 4	5.6	23.33%	0.31%	2.47%	0.0124
Compound 1	Cluster 1	8.5	24.35%	12.01%	0.55%	0.1090
Compound 2	Cluster 1	3.8	7.70%	15.43%	1.14%	0.0001
Compound 3	Cluster 2	7.6	21.20%	10.04%	2.71%	0.0102
Compound 4	Cluster 2	4.1	25.27%	30.57%	5.50%	0.0023
Compound 5	Cluster 3	7.2	22.41%	26.50%	4.34%	0.0183
Compound 6	Cluster 3	8.5; 9.2	13.41%	19.05%	1.52%	0.0003
Compound 7	Cluster 4	3.1; 5.7	16.25%	14.03%	0.65%	0.0022
Compound 8	Cluster 4	8.6	11.57%	3.48%	0.35%	0.0001

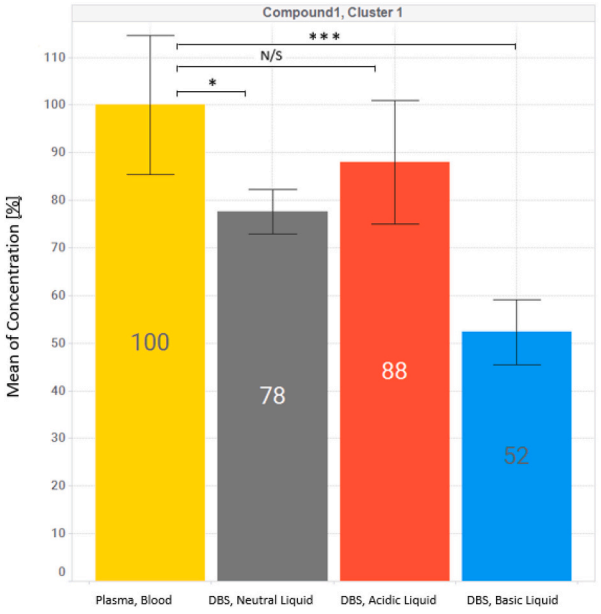
get a complete blood count, while citrate is typically used to assess the blood's ability to clot by reversing anticoagulation. It is worth noting that the test compounds may interact with Citrate or EDTA, potentially forming salts once dried and causing different solubilities.

4.3. Compound pKa and BCS classification

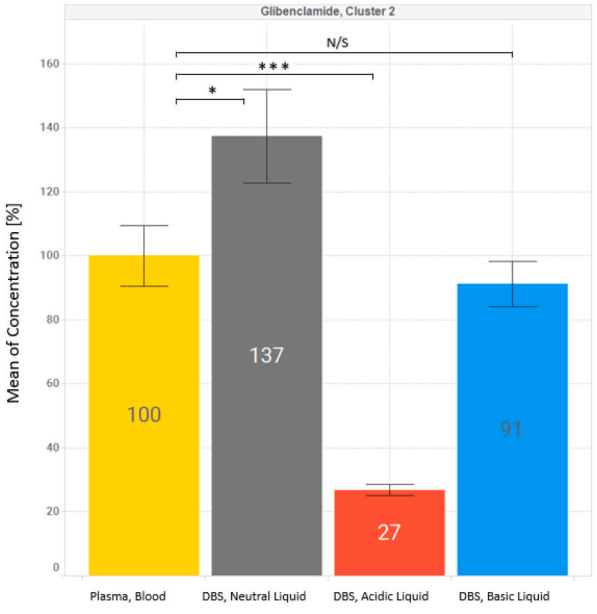
Most compounds were extracted by neutral liquid solutions, but the pKa of each compound may affect the choice of liquid solution to extract the DBS. Charges on some compounds are affected by pH, which can interfere with extraction and solubility. At low pH values (below the pKa), bases become positively charged, while at high pH values (above the pKa), acids become negatively charged. When extracting compounds using neutral liquid solutions, the pKa of each compound should be considered. When the pH is lower than its pKa, a compound with a low pKa donates protons to the solution, while when the pH is higher than its pKa, it acts as a base and accepts protons. Accordingly, the optimal liquid solution should be chosen based on the compound's pKa value. In addition, the concentration and stability of the compound can also be influenced by the pH of the liquid solution. (Settimo, Bellman, & Knegtel, 2014).

4.4. Different compound concentrations

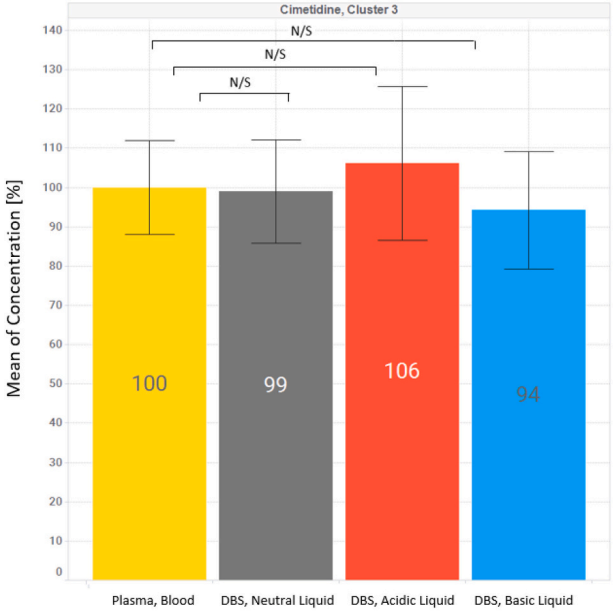
Despite Cp/Cb exhibiting small biological changes with different compound concentrations in samples, DBS compound concentrations act similarly to normal whole liquid blood. Based on the comparisons between our samples when we calculated the Cp/Cb ratio between "Plasma from WHB" and whole blood, we observed equivalent results. Interestingly, the Cp/Cb ratio does not differ with different ranges of



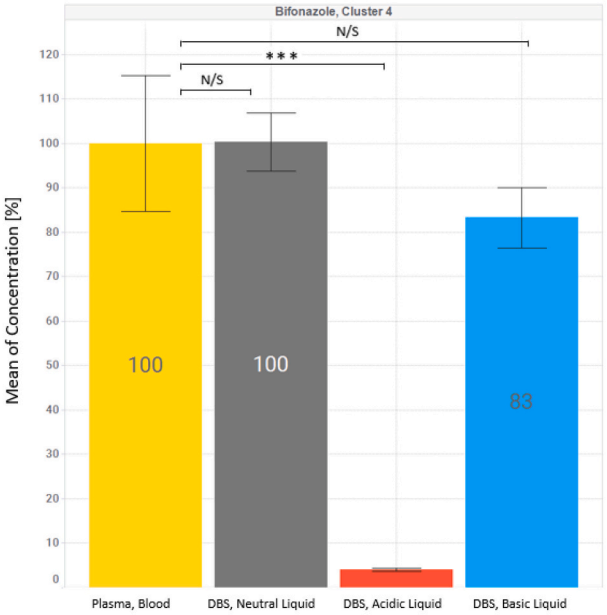
A. Mean (four animals) of percentage of compound concentrations of "Plasma from WHB" and DBS in Metoprolol from BCS1. Values have been normalized by percentages, based on "Plasma from WHB" as the conventional sample of 100 percent.



B. Mean (four animals) of percentage of compound concentrations of "Plasma from WHB" and DBS in Glibenclamide from BCS2. Values have been normalized by percentages, based on "Plasma from WHB".



C. Mean (four animals) of percentage of compound concentrations of "Plasma from WHB" and DBS in cimetidine from BCS3. Values have been normalized by percentages, based on "Plasma from WHB".



D. Mean (four animals) of percentage of compound concentrations of "Plasma from WHB" and DBS in bifonazole from BCS4. Values have been normalized by percentages, based on "Plasma from WHB". With standard deviation as error bars. P-value less than [0.001] appears as '***', between [0.001, 0.01] appears as '**', between [0.01, 0.05] appears as '*', and when p-value > 0.1 , there is N/S.

Fig. 4. Elham: The caption is the text under each part of Figure 4. If not, please let me know to combine all the explanations in one text under all of these figures and put "A,B,C,D" on each part, in word format and send you!

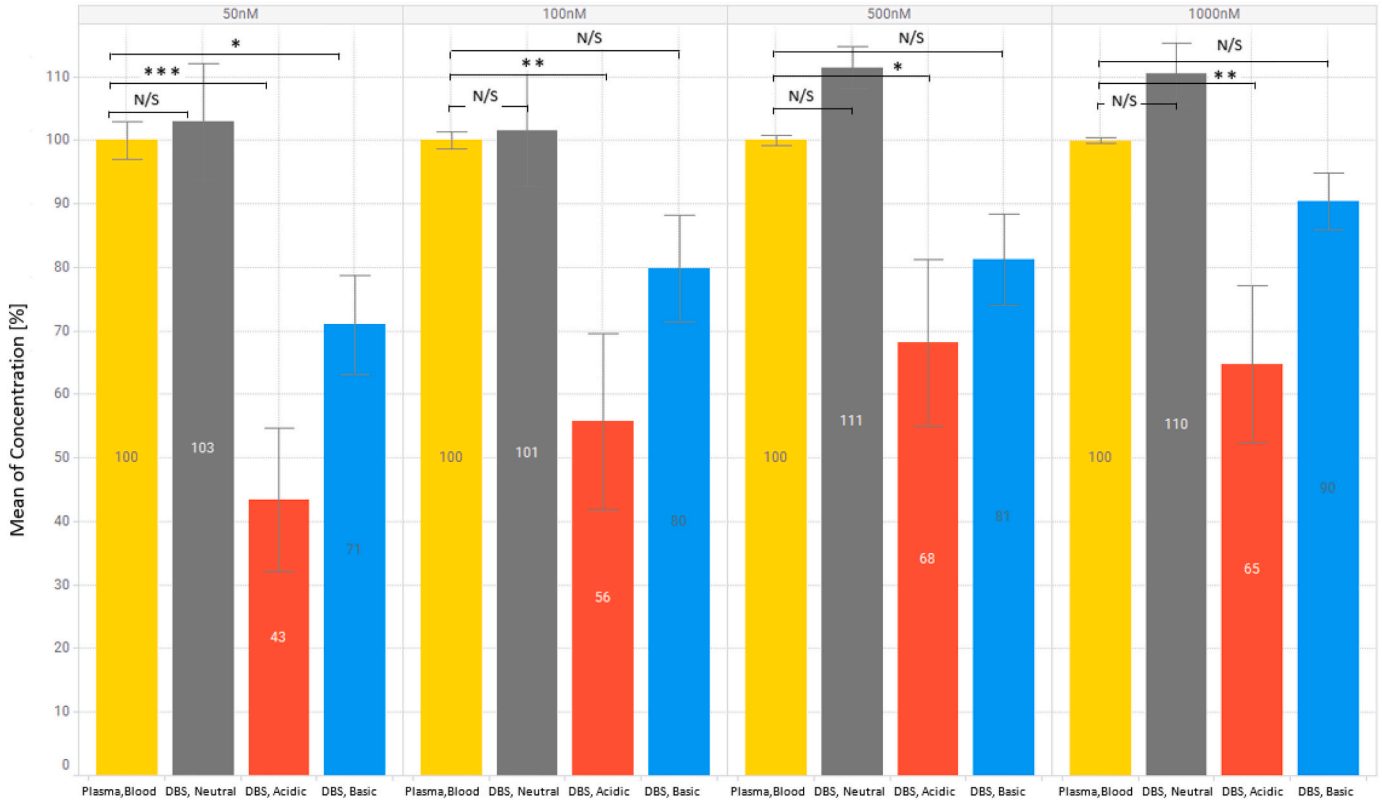


Fig. 5. Mean of all compounds in different Compound concentrations of DBSs compared to "Plasma from WHB" after multiping by their Cp/Cb ratio. Values have been normalized by percentages, based on "Plasma from WHB" of each concentration, as the conventional sample of 100%. *P*-value less than [0.001] appears as '***', between [0.001, 0.01] appears as '**', between [0.01, 0.05] appears as '*', and when *p*-value >0.1, there is N/S.

compound concentration. In the supplementary material, you can find a table of Cp/Cb ratios for each compound concentration and each compound. Based on these results, DBS may provide an effective alternative to traditional methods for obtaining blood for various analytical purposes. (see supplementary material for the table of Cp/Cb ratio for each compound concentration and each compound).

4.5. Comparison with gold standard plasma concentrations

In routine pharmacokinetic studies, plasma derived from freshly drawn venous whole blood is used to determine drug concentrations. Validating the applicability of using DBS in pharmacokinetic research requires the comparison of DBS-derived concentrations with gold standard plasma drug concentrations. When using DBS for sampling, factors such as hematocrit levels, compound stability and extraction efficiency should be considered (Skogstrand et al., 2008; Spooner et al., 2009; Wilhelm et al., 2014). The use of DBS can be facilitated by understanding these factors in order to identify potential limitations and improve its performance for specific compounds or applications.

5. Conclusion

DBS sampling has become increasingly popular in drug development and safety pharmacology studies due to its several advantages, including minimally invasive sampling, small volume requirements, stability of most analytes, and convenient storage and transport (Damon et al., 2018; Skogstrand et al., 2008). By using wearable automatic blood sampling systems (e.g., Fluispotter), dried blood samples (DBS) can be collected with minimal disruption to the physiological parameters of test subjects, thereby improving PK/PD modelling accuracy.

With automatic blood samplers attached to animals and programmed to collect blood samples at predetermined times, serial samples can be collected accurately and precisely by storing dried blood drops on filter paper. This method provides several advantages related to animal ethics and welfare. During blood collection, the device's automatic collection reduces human interaction, minimizing physiological parameters and improving measurement accuracy. Moreover, this noninvasive device allows for more blood sampling time points without distracting the animal or the study.

In this way, drug effects can be better understood, and PK/PD models can be developed more accurately. Overall, the use of wearable automatic blood sampling systems has great potential to improve the accuracy and ethical standards of safety pharmacology studies.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgements

Elham is ESR-fellow of the INSPIRE European Training Network. INSPIRE receives funding from the EU Horizon 2020 Research and Innovation programme, under the Marie Skłodowska Curie, GA 858070.

Thanks to all members of the General Pharmacology group and Bioanalysis team of the Drug Discovery Sciences department at Boehringer Ingelheim Pharma GmbH & Co KG for their support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.vascn.2023.107296.

References

Adhikari, K. B., Rohde, M., Velschow, S., Feldt-Rasmussen, U., Johannesen, J., & Johnsen, A. H. (2020). Fluispotter, a novel automated and wearable device for accurate volume serial dried blood spot sampling. *Bioanalysis.*. https://doi.org/10.4155/bio-2020-0048

Ahmad, S. A., She, Z., & Kraatz, H. (2019). Electrochemical studies of human nAChR a7 subunit phosphorylation by kinases PKA, PKC and Src. *Analytical Biochemistry, 574*, 46–56. https://doi.org/10.1016/j.ab.2019.03.012

Alizadeh, E. A., Trautmann, T., Krause, F., Knoeferl, B., Guns, P., De Meyer, G., … Markert, M. (2023). The impact of environmental and biological factors on the resting heart rate of dogs as assessed using 20 years of data from safety pharmacology studies. *Journal of Pharmacological and Toxicological Methods, 121*, Article 107263. https://doi.org/10.1016/j.vascn.2023.107263

Burgess, R. R. (2009). Chapter 20 protein precipitation techniques. In *Methods in enzymology* (pp. 331–342). Academic Press. https://doi.org/10.1016/s0076-6879(09)63020-2.

Chavda, H. V., Patel, C. K., & Anand, I. S. (2010). Biopharmaceutics classification system. *Systematic Reviews in Pharmacy, 1*(1), 62. https://doi.org/10.4103/0975-8453.59514

Chen, B., Fong, J., & Chiang, C. (1999). Effect of different anticoagulant, underfilling of blood sample and storage stability on selected hemogram. *PubMed, 15*(2), 87–93. https://pubmed.ncbi.nlm.nih.gov/10089718.

Choi, R., Park, H., Oh, J. H., Lee, K., Song, J., & Lee, S. (2019). Dried blood spot multiplexed steroid profiling using liquid chromatography tandem mass spectrometry in Korean neonates. *Annals of Laboratory Medicine, 39*(3), 263–270. https://doi.org/10.3343/alm.2019.39.3.263

Damon, D. E., Yin, M., Allen, D. M., Maher, Y. S., Tanny, C. J., Oyola-Reynoso, S., … Badu-Tawiah, A. K. (2018). Dried blood spheroids for dry-state room temperature stabilization of microliter blood samples. *Analytical Chemistry, 90*(15), 9353–9358. https://doi.org/10.1021/acs.analchem.8b01962

Eldjarn, C., Andersen, M. W., Forfang, R. M., Hagve, S. E., & Rootwelt, K. (2011). Experiences with citrate rather than heparin as an anticoagulant for 18F-FDG labeling of leukocytes. *Journal of Nuclear Medicine Technology*. https://doi.org/10.2967/jnmt.110.085464

EMA/CHMP. (2016). Guideline on the use of pharmacokinetics and pharmacodynamics in the development of antimicrobial medicinal products. In *European Medicines Agency, EMA/CHMP/594085/2015*. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-use-pharmacokinetics-pharmacodynamics-development-antimicrobial-medicinal-products_en.pdf.

Food and Drug Administration. (2022). Population pharmacokinetics guidance for industry. U.S. Department of Health And Human Services Food And Drug Administration. https://www.fda.gov/media/128793/download.

Gross, K. C., Seybold, P. G., & Hadad, C. M. (2002). Comparison of different atomic charge schemes for predicting pKa variations in substituted anilines and phenols. *International Journal of Quantum Chemistry, 90*(1), 445–458. https://doi.org/10.1002/qua.10108

Guns, P., Guth, B., Braam, S. R., Kosmidis, G., Matsa, E., Delaunois, A., … Valentin, J. (2020). INSPIRE: A European training network to foster research and training in cardiovascular safety pharmacology. *Journal of Pharmacological and Toxicological Methods, 105*, Article 106889. https://doi.org/10.1016/j.vascn.2020.106889

Hang, J., Vento, T. J., Norby, E. A., Jarman, R. G., Keiser, P. B., Kuschner, R. A., & Binn, L. N. (2017). Adenovirus type 4 respiratory infections with a concurrent outbreak of coxsackievirus A21 among United States Army basic trainees, a retrospective viral etiology study using next-generation sequencing. *Journal of Medical Virology, 89*(8), 1387–1394. https://doi.org/10.1002/jmv.24792

Hennø, L. T., Storjord, E., Christiansen, D., Bergseth, G., Ludviksen, J. K., Fure, H., … Brekke, O. (2017). Effect of the anticoagulant, storage time and temperature of blood samples on the concentrations of 27 multiplex assayed cytokines – Consequences for defining reference values in healthy humans. *Cytokine, 97*, 86–95. https://doi.org/10.1016/j.cyto.2017.05.014

Keustermans, G., Hoeks, S. E., Meerding, J., Prakken, B. J., & De Jager, W. (2013). Cytokine assays: An assessment of the preparation and treatment of blood and tissue samples. *Methods, 61*(1), 10–17. https://doi.org/10.1016/j.ymeth.2013.04.005

Lee, G. M., & Arepally, G. M. (2012). Anticoagulation techniques in apheresis: From heparin to citrate and beyond. *Journal of Clinical Apheresis, 27*(3), 117–125. https://doi.org/10.1002/jca.21222

Morgan, P. S., Brown, D. G., Lennard, S. N., Anderton, M. J., Barrett, J. C., Eriksson, U., … Pangalos, M. N. (2018). Impact of a five-dimensional framework on R&D productivity at AstraZeneca. *Nature Reviews Drug Discovery, 17*(3), 167–181. https://doi.org/10.1038/nrd.2017.244

Musteata, F. M., De Lannoy, I., Gien, B., & Pawliszyn, J. (2008). Blood sampling without blood draws for in vivo pharmacokinetic studies in rats. *Journal of Pharmaceutical and Biomedical Analysis, 47*(4–5), 907–912. https://doi.org/10.1016/j.jpba.2008.03.028

Ollerenshaw, J., Schrøder, M., & Velschow, S. (2022). A novel device for serial venous blood sampling in a canine model. *Journal of Pharmacological and Toxicological Methods, 107155*. https://doi.org/10.1016/j.vascn.2022.107155

Peck, C. C., Barr, W. B., Benet, L. Z., Collins, J. M., Desjardins, R., Furst, D. E., … Yacobi, A. (1992). Opportunities for integration of pharmacokinetics, pharmacodynamics, and toxicokinetics in rational drug development. *Clinical Pharmacology and Therapeutics, 51*(4), 465–473. https://doi.org/10.1038/clpt.1992.47

Rajman, I. (2008). PK/PD modelling and simulations: Utility in drug development. *Drug Discovery Today, 13*(7–8), 341–346. https://doi.org/10.1016/j.drudis.2008.01.003

Reddy, B. B. K., & Karunakar, A. (2011). Biopharmaceutics classification system: A regulatory approach. *Dissolution Technologies, 18*(1), 31–37. https://doi.org/10.14227/dt180111p31

Settimo, L., Bellman, K., & Knegtel, R. (2014). Comparison of the accuracy of experimental and predicted pKa values of basic and acidic compounds. *Pharmaceutical Research, 31*(4), 1082–1095. https://doi.org/10.1007/s11095-013-1232-z

Shieh, Y., Liu, G., Wu, H., & Lee, C. (2007). Effects of polarity and pH on the solubility of acid-treated carbon nanotubes in different media. *Carbon, 45*(9), 1880–1890. https://doi.org/10.1016/j.carbon.2007.04.028

Skogstrand, K., Ekelund, C. K., Thorsen, P., Vogel, I., Jacobsson, B., Nørgaard-Pedersen, B., & Hougaard, D. M. (2008). Effects of blood sample handling procedures on measurable inflammatory markers in plasma, serum and dried blood spot samples. *Journal of Immunological Methods, 336*(1), 78–84. https://doi.org/10.1016/j.jim.2008.04.006

Spooner, N., Lad, R., & Barfield, M. (2009). Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: Considerations for the validation of a quantitative bioanalytical method. *Analytical Chemistry, 81*(4), 1557–1563. https://doi.org/10.1021/ac8022839

Van Der Graaf, P. H., & Benson, N. (2011). Systems pharmacology: Bridging systems biology and pharmacokinetics-pharmacodynamics (PKPD) in drug discovery and development. *Pharmaceutical Research, 28*(7), 1460–1464. https://doi.org/10.1007/s11095-011-0467-9

Van Groen, B. D., Reddy, V. P., Badée, J., Olivares-Morales, A., Johnson, T. N., Nicolaï, J., … De Zwart, L. (2020). Pediatric pharmacokinetics and dose predictions: A report of a satellite meeting to the 10th juvenile toxicity symposium. *Clinical and Translational Science, 14*(1), 29–35. https://doi.org/10.1111/cts.12843

Velghe, S., Delahaye, L., & Stove, C. P. (2019). Is the hematocrit still an issue in quantitative dried blood spot analysis? *Journal of Pharmaceutical and Biomedical Analysis, 163*, 188–196. https://doi.org/10.1016/j.jpba.2018.10.010

Wilhelm, A. J., Burger, J. C. G. D., & Swart, E. L. (2014). Therapeutic drug monitoring by dried blood spot: Progress to date and future directions. *SpringerLink, 53*(11), 961–973. https://doi.org/10.1007/s40262-014-0177-7

Workman, P. (2002). Challenges of PK/PD measurements in modern drug development. *European Journal of Cancer, 38*(16), 2189–2193. https://doi.org/10.1016/s0959-8049(02)00395-7